Paving the way for pulmonary influenza vaccines

Tomar, Jasmine

Chapter 7

Summary, concluding remarks, and perspectives

Summary

Vaccination against influenza is considered to be the cornerstone in the prevention and control of influenza outbreaks[1,2]. However, except for Flumist®, influenza vaccines are currently administered by injection, which induces sub-optimal immune responses. In this respect, an inhalable influenza vaccine could be advantageous as the respiratory tract has abundant immune cells and is the portal of influenza virus entry[3,4]. Despite that, several issues related to administration of influenza vaccines via the respiratory tract are still unaddressed and need further investigation.

In this thesis, we explored two different approaches to enhance immunogenicity of influenza vaccine candidates upon administration via the respiratory tract. In the first approach, the site of deposition in the respiratory tract that elicits optimal immune responses was investigated. The second approach deals with the adjuvantation of influenza vaccine candidate; whether or not adjuvantation has the potential to augment immune responses which ultimately leads to enhanced protective efficacy.

In **Chapter 2**, we discuss current developments in the field of dry influenza vaccines. Currently, influenza vaccine formulations are only available in liquid state. The liquid state has the disadvantage that these formulations need to be stored and distributed refrigerated, the so-called ‘‘cold chain’’, so as to prevent antigen degradation at high temperatures. Drying influenza vaccine with suitable excipients restricts molecular mobility and thus preserves its conformational and chemical integrity during subsequent storage. It thereby alleviates the need of cold chain and aids stockpiling; thus facilitating rapid use in case of pandemic outbreaks. Dry influenza vaccines for (pre)-clinical research are commonly prepared by spray drying, spray freeze drying, air drying or vacuum drying. Influenza vaccines in the dry state have the concomitant advantage that they can be formulated as non-parenteral, patient friendly dosage forms, for example, powders for epidermal, nasal or pulmonary administration, tablets for oral administration or microneedles for transdermal administration. Furthermore, having the influenza vaccine in dry state and its administration via these alternative routes has shown to induce comparable or better immune responses than the traditional parenteral administration **(Chapter 2).** Thus, dry influenza vaccines have shown enhanced stability, better efficacy and are convenient to use due to their administration via non-parenteral routes. Therefore, these dry influenza vaccines have

huge potential and are a promising alternative to currently available influenza vaccine formulations; thus encouraging further developments in this field.

In **Chapter 3,** pulmonary administration of a whole inactivated influenza virus powder vaccine formulation was compared to a liquid formulation in a cotton rat model with respect to the site of deposition in the respiratory tract, immunogenicity and protective efficacy. Spray freeze drying was used to produce dry particles in a size range suitable for inhalation (1–5 μm). Despite the appropriate size range, we found that powder formulation was predominantly deposited in the trachea of the animals whereas liquid influenza vaccine formulation had lung lobes as their deposition site. Deposition of powders in the trachea was attributed to; a) poor de-agglomerating property of the pre-clinical commercially available device (now discontinued), insufflator (Penn-Century, USA), thus producing particles of large size; b) and due to the return flow of air (containing powder particles). Deposition of liquids in the lung lobes was most likely the result of a simple ''dripping down effect''. Though in the immunogenicity studies, liquid influenza vaccine formulation had induced significantly higher immune responses than the powder formulation, the overall protective efficacy of both these formulations against the clinical isolate of live virus was found to be comparable. The differences in immune responses were found to be due to incomplete dosing of the powder formulation (exhalation and residual amount in the insufflator) rather than the differences in deposition site. Thus, cotton rats are successful as a model for pulmonary vaccination and site of influenza deposition seems to be of minor relevance in inducing protective immunity.

Due to the limitations of the insufflator observed in **Chapter 3,** another pre-clinical in-house built delivery device, aerosol generator, was used to target vaccine powders deep into the lungs. Besides influenza, in **Chapter 4**, we investigated whether site of antigen deposition also holds minor relevance for vaccines against diseases that do not spread via the respiratory tract, for example, hepatitis B. Fluorescently labeled vaccine formulations were spray dried to produce dry powder formulations which were then targeted to trachea/central airways of mice by using the insufflator and to the deep lungs by using the aerosol generator. Immunogenicity studies in mice showed that deposition of dry influenza subunit vaccine formulation into trachea/central airways or into deep lungs did not have any impact on the magnitude of immune responses. For influenza, sialic acid receptors present in the upper part of

respiratory tract might play a role in the uptake of vaccine to induce an immune response, thus circumventing mucociliary clearance of the vaccine. However, for hepatitis B surface antigen, only deep lungs deposition led to the induction of considerable immune responses whereas trachea/central airways deposition did not. No hepatitis B specific receptors are present in any part of the respiratory tract. As a result, hepatitis B vaccine deposited in the trachea/central airways would most likely have been removed by mucociliary clearance. Nevertheless, deep lung deposition of hepatitis B vaccine was speculated to prolong the residence time of the antigen along with diffusion of the antigen to lung draining lymph nodes for better interaction with immune cells. Thus, influence of site of deposition is dependent upon the type of antigen and should be taken into account so as to enhance the efficacy of respiratory tract administered vaccines.

In **Chapter 5**, we utilized a second approach of enhancing the immunogenicity of respiratory tract delivered whole inactivated influenza virus vaccine i.e. by adjuvantation. In this study, we investigated Advax™, a delta isoform of inulin as a mucosal adjuvant for influenza vaccine administered either as liquid or dry powder influenza vaccine formulations by intranasal or pulmonary route in mice. The physical and biological properties of influenza vaccine and Advax were found to be preserved both by admixing Advax with influenza vaccine and by spray freeze drying to produce dry powder particles. *In-vivo* immunogenicity studies in mice by intranasal or pulmonary administration revealed that Advax adjuvantation has the potential to enhance both humoral (systemic and mucosal) and cellular immune responses both as liquids and as dry powders. In addition, pulmonary administration of Advax adjuvanted influenza vaccine also led to an increment in the frequency of memory B cells along with an increase in the expression of lung localization factors. A moderate enhancement could also be seen in T cell memory cells as well as the percentage of localization factors on these memory T cells. Lastly, a single dose of Advax adjuvanted influenza vaccine not only protected mice against lethal dose of homologous live virus but also did not lead to the development of any clinical symptoms with a clinical sickness score of 0. On the contrary, mice immunized with influenza vaccine alone had to be euthanized due to the progression of sickness with a score of 6, 8–9 days post challenge. Hence, Advax adjuvant has the potential to augment influenza vaccine induced immune responses with a pronounced effect on B cell memory and complete protection post challenge.

In **Chapter 6**, we further developed adjuvanted dry whole inactivated influenza virus vaccine formulations and investigated their potential to protect chickens against lethal dose of live avian influenza virus. Spray freeze drying was used to produce Advax or Bacterium-like particles (BLP) adjuvanted dry powder particles in a size range that is suitable for inhalation by chickens. The physical and biological characteristics of influenza vaccine and both the adjuvants remained unaltered during the spray freeze drying process. In order to investigate the potential of these adjuvants in a chicken model, initially, these dry powder formulations were delivered directly at the syrinx of chickens (active administration/inhalation). Active inhalation studies revealed that BLP and Advax boost either systemic or mucosal immune responses or both. In a second series of experiments, chickens were placed in a box in which dry powder vaccine formulations were aerosolized, chickens were allowed to inhale these aerosolized powder particles for a certain time period (passive administration/ inhalation). Passive inhalation of adjuvanted dry powder formulations augmented systemic immune responses to a substantial extent as compared to non-adjuvanted formulation. Moreover, passive administration of either non-adjuvanted or adjuvanted vaccine formulations conferred protective immunity in chickens as evident from their complete survival post lethal challenge with live influenza virus. In addition, no lung virus titers could be detected in any of the vaccinated animals except for two out of six animals in the Advax adjuvanted group. Conclusively, passive inhalation seems to be a feasible and effective method of mass vaccination that has the potential to protect chickens from morbidity and mortality against avian influenza virus.

Concluding remarks and perspectives

Vaccination via the respiratory tract is a feasible, effective and convenient alternative to conventional parenteral vaccination. Though pre-clinical studies have shown it to be an attractive alternative, the only marketed product for respiratory tract administration is Flumist®, currently, used via the intranasal route. No such product is yet in the market for pulmonary administration. In this thesis, we have focused our research on two important aspects that can lead to efficacious respiratory tract administration, in particular, via the pulmonary route.

Currently available influenza vaccine formulations for parenteral or intranasal administration pose major instability issues outside the cold chain. The requirement

of cold chain not only adds up to the costs of vaccination and thus economic burden but also tons of vaccine doses are wasted every year due to improper storage and distribution. Hence, from the past decade, the current research is focused on the development of dry influenza vaccines for non-parenteral administration. Bringing these liquid vaccine candidates into dry state using appropriate excipients and drying techniques can render them into stable formulations, which do not depend on the cold chain. Hence, these dry influenza vaccines are preferred and are expected to draw more attention in the future.

Future research should focus on clinical trials to assess the safety and efficacy of dry powder influenza vaccine formulations. Some clinical trials conducted in the past with liquid influenza vaccine formulations have reported pulmonary administration to be a safe route which has comparable potential as parenteral route in terms of protection[5,6]. However, dry influenza vaccines have still not landed up in the clinic. Thus far the only inhalable dry powder vaccine clinically tested was a measles vaccine formulation, which was well tolerated in subjects[7]. The challenge now remains to assess how the safety and efficacy aspects of this route would be governed by the pulmonary administration of dry powder influenza vaccines.

The starting point for the clinical studies should be the development of effective dry powder influenza formulations that can be administered by suitable devices. Although pre-clinical studies have shown that after respiratory tract administration of non-adjuvanted influenza vaccine formulations substantial local IgA titers were induced, the overall immunogenicity was poor[8,9]. Effective mucosal adjuvants are therefore needed, which most importantly, should be safe. Severe cases of Bell's palsy reported in subjects vaccinated with intranasal influenza virosomal vaccine (*NasalFlu*) adjuvanted with mutant of *Escherichia coli* heat labile toxin, LTK63, not only led to the withdrawal of this product from the market but also raised serious concerns on safety issues associated with mucosal adjuvants in general[10]. Currently, limited (pre)-clinical safety data are available on the two mucosal adjuvants studied in this thesis; a) parenteral administration of influenza vaccines with Advax[11,12] and b) intranasal administration of bacterium like particles adjuvanted influenza vaccines[13]. Forthcoming research in the area of pulmonary vaccine delivery should focus on extensive analysis of safety and toxicity of mucosal adjuvants both in animal models and clinical trials. Besides safety, more research is needed on the efficacy of

adjuvanted formulations upon respiratory tract administration. For example, Advax adjuvant was found to enhance memory responses upon pulmonary administration with influenza vaccine in a mouse model. In the future studies, it should be investigated whether or not these enhanced memory responses induced upon Advax adjuvantation would provide long term protection against influenza.

For the dispersion of dry powder formulations, effective disposable dry powder inhalers like the Twincer, Torus and many more are available for use in clinical studies[14], however, the lack of suitable powder delivery devices in pre-clinical research warrants further developments in this field. The most commonly used device for powder administration in pre-clinical research, insufflator, suffers from certain limitations and thus dry powder vaccines (influenza and hepatitis B) were found to be deposited in the trachea/central airways of pre-clinical animal models. Another newly marketed device, PreciseInhale, was found to cause less tracheal deposition as compared to the insufflator; thus it might be a suitable alternative to the insufflator[15]. However, the high pressure pulses used in the PreciseInhale system for the dispersion of dry powders were found to be detrimental to powders as these caused crystallization of amorphous spray dried products[16]. An amorphous sugar matrix is needed for the stabilization of vaccines. Crystallization of the sugar due to high pressure poses mechanical stress to the stabilized vaccine, thus, leading to the deterioration of the vaccine. In addition, the dose that reached the lungs of animals was extremely low with PreciseInhale (1–4% of the loaded product). Likewise, the in-house built aerosol generator was also found to have low dosing efficiency (0.3% of the loaded product), thus steps should be taken for improvement. Reducing the volume of the aerosol chamber so as to increase the concentration of the aerosolized vaccine or increasing the number of ports so that multiple animals can simultaneously inhale the aerosolized vaccine would be suitable ways to enhance the dosing efficiency. Despite of dosing issues, the aerosol generator might be a better choice than the insufflator or PreciseInhale. Insufflator preserves the amorphous state of the product but causes tracheal deposition, whereas with PreciseInhale, deep lung deposition is possible but the amorphous state of the product is compromised. The aerosol generator was not only found to preserve the amorphous state of the spray dried product but also caused deep lung deposition, the two major limitations determined with these two commercial devices. Whether deep lung deposition is of minor relevance or major importance for a number of vaccine candidates, still needs to be

investigated in detail. For vaccine candidates like influenza, deep lung deposition did not seem to be crucial for the protective efficacy against virus challenge. Likewise, for tuberculosis and measles vaccine, deposition in the upper airways has shown to induce considerable immune responses[17]. However, the same conclusion cannot be generalized for each vaccine candidate administered via the respiratory tract. For example, hepatitis B vaccine when deposited in the trachea/central airways upon dispersion from the insufflator did not induce any immune response at all. Deep lung deposition of hepatitis B vaccine upon delivery from newly developed in-house built aerosol generator was found to be favorable in inducing immunity. Hence, for each vaccine candidate, the optimal site of antigen deposition in the respiratory tract should be investigated in pre-clinical studies before proceeding to clinical research. In addition, it should also be investigated as to why some vaccines need deep lung targeting (hepatitis B) whereas other vaccines against pathogens like influenza, measles and tuberculosis can do without it. To our knowledge, it is still unclear whether this finding has some relation with the presence of certain receptors in the respiratory tract (sialic acid receptors for influenza, CD46 for measles and pattern recognition receptors for tuberculosis), which might play a role in the uptake of vaccines to induce an immune response as they do in the uptake of viruses. In this respect, vaccine candidates for which no specific receptors are present in the respiratory tract (e.g. hepatitis B), might rely on alternative mechanisms such as passive transport of the antigen to lymph nodes where interaction with abundant immune cells will eventually lead to an immune response. From our studies and literature, we know that for air borne diseases such as influenza, measles and tuberculosis, deep lung deposition is not required, however, for non-air borne diseases such as hepatitis B, deep lung deposition is must. Hence, more research is needed in this area to investigate whether this conclusion can be generalized for vaccine candidates against air-borne diseases such as pertussis, respiratory syncytial infection and against non air-borne infectious diseases such as cholera, typhoid.

Apart from having suitable formulations that deposit in the appropriate region of the respiratory tract, another important aspect is to prevent the transmission of avian influenza (bird flu) to the human population. For this, effective non-adjuvanted or adjuvanted influenza formulations were inhaled by chickens during normal breathing of the animals (passive administration). Even the non-adjuvanted influenza formulation was able to provide complete protection in an improved passive inhalation set-up

(improved as compared to our previous study). This suggests that in future studies the use of adjuvants might not be necessary but can provide dose sparing effects as large vaccine doses will be needed for field studies. The next step would be to apply this passive inhalation concept on a large scale, for example, in a field with a number of chickens. However, factors like the area of the field, delivery devices for dispersion of these powders and settling rate after dispersion should be taken into account.

Conclusively, clinical studies would only become successful if all these factors associated with pulmonary vaccination are investigated in suitable pre-clinical animal models. In this thesis, we explored mice, cotton rats and chickens as influenza research models. Though mice are the most widely used models for influenza research, a number of disadvantages are associated with them. For example, pulmonary administration in mice is complicated because of their relatively narrow trachea. In addition, mouse adapted influenza strains are needed for challenge studies and the disease progression is asymptomatic. On the contrary, cotton rats are less prone to mechanical damage during pulmonary delivery, can be challenged with a clinical isolate of influenza virus and disease progression is symptomatic after challenge[18]. Bigger animal models such as ferrets and guinea pigs are also more suitable as they can be challenged with a human virus isolate as well[19]. Chickens used in this thesis were not intended as animal model for humans, however, the restriction of avian influenza in poultry would have a significant positive impact on the human population.

Hence, different factors like efficacy and safety of newly developed formulations, delivery devices for dispersion and site of deposition upon pulmonary administration should be investigated in suitable pre-clinical animal models.

References

[1] Houser K, Subbarao K. Influenza vaccines: Challenges and solutions. *Cell Host Microbe.* 2015; 17: 295–300.

[2] Jennings LC, Monto AS, Chan PK, *et al.* Stockpiling prepandemic influenza vaccines: a new cornerstone of pandemic preparedness plans. *Lancet Infect. Dis.* 2008; 8: 650–58.

[3] Amorij JP, Hinrichs WLJ, Frijlink HW, *et al.* Needle-free influenza vaccination. *Lancet Infect. Dis.* 2010; 10: 699–711.

[4] Tomar J, Born PA, Frijlink HW, *et al.* Dry influenza vaccines: towards a stable, effective and convenient alternative to conventional parenteral influenza vaccination. *Expert Rev. Vaccines.* 2016; 15: 1431–447.

[5] Waldman RH, Mann J, Small PA. Immunization Against Influenza, Prevention of Illness in Man by Aerosolized Inactivated Vaccine. *JAMA.* 1969; 207:520–24.

[6] Waldman RH, Wood SH, Torres EJ, *et al.* Influenza antibody response following aerosal administration of inactivated virus. *Am J Epidemiol.* 1970; 91: 574–85.

[7] Agarkhedkar S, Kulkarni PS, Winston S, *et al.* Safety and immunogenicity of dry powder measles vaccine administered by inhalation: A randomized controlled Phase I clinical trial. *Vaccine.* 2014; 32: 6791–797.

[8] Audouy SAL, van der Schaaf G, Hinrichs WLJ, *et al.* Development of a dried influenza whole inactivated virus vaccine for pulmonary immunization. *Vaccine.* 2011; 29: 4345–352.

[9] Patil HP, Murugappan S, ter Veer W, *et al.* Evaluation of monophosphoryl lipid A as adjuvant for pulmonary delivered influenza vaccine. *J. Control. Release.* 2014; 174: 51–62.

[10] Lewis DJM, Huo Z, Barnett S, *et al.* Transient facial nerve paralysis (Bell's palsy) following intranasal delivery of a genetically detoxified mutant of Escherichia coli Heat Labile Toxin. *PLoS One.* 2009; 4: 1–5.

[11] Gordon DL, Sajkov D, Woodman RJ, *et al.* Randomized clinical trial of immunogenicity and safety of a recombinant H1N1/2009 pandemic influenza vaccine containing Advax™ polysaccharide adjuvant. *Vaccine.* 2012; 30: 5407–416.

[12] Gordon D, Kelley P, Heinzel S, *et al.* Immunogenicity and safety of Advax™ , a novel polysaccharide adjuvant based on delta inulin, when formulated with hepatitis B surface antigen; a randomized controlled Phase 1 study. *Vaccine.* 2014; 32: 6469–477.

[13] Budimir N, Huckriede A, Meijerhof T, *et al.* Induction of heterosubtypic cross-protection against influenza by a whole inactivated virus vaccine: The role of viral membrane fusion activity. *PLoS One.* 2012; 7: e30898.

[14] Tonnis WF, Lexmond AJ, Frijlink HW, *et al.* Devices and formulations for pulmonary vaccination. *Expert Opin. Drug Deliv.* 2013; 10: 1383–397.

[15] Fioni A, Selg E CV, *et al.* Investigation of Lung Pharmacokinetic of the Novel PDE4 Inhibitor CHF6001 in Preclinical Models: Evaluation of the PreciseInhale Technology. *J. Aerosol Med. Pulm. Drug Deliv.* 2018; 31: 61–70.

[16] Lexmond AJ, Keir S, Terakosolphan W, *et al.* A novel method for studying airway hyperresponsiveness in allergic guinea pigs in vivo using the PreciseInhale system for delivery of dry powder aerosols. *Drug Deliv. Transl. Res.* 2018; 8: 760–69.

[17] Todoroff J, Ucakar B, Inglese M, *et al.* Targeting the deep lungs, Poloxamer 407 and a CpG oligonucleotide optimize immune responses to Mycobacterium tuberculosis antigen 85A following pulmonary delivery. *Eur. J. Pharm. Biopharm.* 2013; 84: 40–48.

[18] Bhide Y, Tomar J, Dong W, *et al.* Pulmonary delivery of influenza vaccine formulations in cotton rats: site of deposition plays a minor role in the protective efficacy against clinical isolate of H1N1pdm virus. *Drug Deliv.* 2018; 25: 533–45.

[19] Bouvier NM, Lowen AC. Animal models for influenza virus pathogenesis and transmission. *Viruses.* 2010; 2: 1530–563.